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Niche signaling promotes stem cell survival in the *Drosophila* testis via the JAK–STAT target DIAP1

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ABSTRACT

Tissue-specific stem cells are thought to resist environmental insults better than their differentiating progeny, but this resistance varies from one tissue to another, and the underlying mechanisms are not well-understood. Here, we use the *Drosophila* testis as a model system to study the regulation of cell death within an intact niche. This niche contains sperm-producing germline stem cells (GSCs) and accompanying somatic cyst stem cells (or CySCs). Although many signals are known to promote stem cell self-renewal in this tissue, including the highly conserved JAK–STAT pathway, the response of these stem cells to potential death-inducing signals, and factors promoting stem cell survival, have not been characterized. Here we find that both GSCs and CySCs resist cell death better than their differentiating progeny, under normal laboratory conditions and in response to potential death-inducing stimuli such as irradiation or starvation. To ask what might be promoting stem cell survival, we characterized the role of the anti-apoptotic gene *Drosophila* inhibitor of apoptosis 1 (*diap1*) in testis stem cells. DIAP1 protein is enriched in the GSCs and CySCs and is a JAK–STAT target. *diap1* is necessary for survival of both GSCs and CySCs, and ectopic up-regulation of DIAP1 in somatic cyst cells is sufficient to non-autonomously rescue stress-induced cell death in adjacent differentiating germ cells (spermatogonia). Altogether, our results show that niche signals can promote stem cell survival by up-regulation of highly conserved anti-apoptotic proteins, and suggest that this strategy may underlie the ability of stem cells to resist death more generally.

Introduction

Adult stem cells maintain tissues by producing both stem cells and differentiated daughters that replenish lost or dying cells to ensure the integrity of the entire organism (Rossi et al., 2008). Stem cells reside in specific microenvironments termed niches, which produce signals that maintain stem cell populations (Jones and Wagers, 2008; Spradling et al., 2001). All cells within an organism, including stem cells, can be challenged by cellular stresses that lead to cell death (Sancar et al., 2004). These stresses can arise endogenously, such as from the accumulation of reactive oxygen species or errors during DNA replication, or exogenously from environmental insults including radiation or poor nutrition (Drummond-Barbosa and Spradling, 2001; Sancar et al., 2004).

In general, stem cells are thought to be more resistant to cell death than their differentiating progeny (Mandal et al., 2011). However, this is not the case for all stem cells. The response of stem cells and their progeny to ionizing radiation as a source of stress has been well characterized for several adult mammalian tissues (Blanpain et al., 2011). While stem cells in some tissues are more radio-resistant than their differentiating progeny, stem cells in other tissues are highly susceptible to terminal differentiation or death upon ionizing radiation (Blanpain et al., 2011; Liu et al., 2014). For example, Hematopoietic stem cells (HSCs), mammary stem cells, hair follicle bulge stem cells (BSCs) (Sotiropoulou et al., 2010) and keratinocyte stem cells in the adult mouse are much more radio-resistant than their differentiating progeny (Liu et al., 2014; Rachidi et al., 2007). In contrast, melanocyte stem cells, which reside in the same niche as BSCs, are not radio-resistant. The same dose of radiation that is tolerated by BSCs induces massive terminal differentiation in melanocyte stem cells (Blanpain et al., 2011; Inomata et al., 2009; Insinga et al., 2014; Sotiropoulou et al., 2010). Mammalian intestinal stem cells (ISCs) are also extremely sensitive to radiation: radiation doses lower than those tolerated by BSCs induce ISC death (Blanpain et al., 2011; Sotiropoulou et al., 2010). In cases of radio-sensitive stem cells, radiation-induced DNA damage triggers the highly conserved process of programmed cell death (PCD).

Programmed cell death (PCD) occurs via three canonical pathways: necrosis, autophagy and apoptosis (Fuchs and Steller,

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2011). Necrosis involves swelling and rupture of the cellular organelles and is characterized by an increase in intracellular Ca^{2+}, reactive oxygen species and acidity (McCall, 2010). Autophagy involves engulfment of subcellular components by autophagosomes, which fuse with lysosomes to form autophagolysosomes where the engulfed cytoplasmic material is degraded (Fuchs and Steller, 2011). Apoptosis (or type I PCD), the most common and well-studied form of PCD, is characterized by cell shrinkage, nuclear condensation and membrane blebbing. Apoptosis involves the activation of cysteine proteases called caspases (Kerr et al., 1972; Ouyang et al., 2012). Initiator caspases are activated in response to apoptotic stimuli, and in turn, they cleave and activate

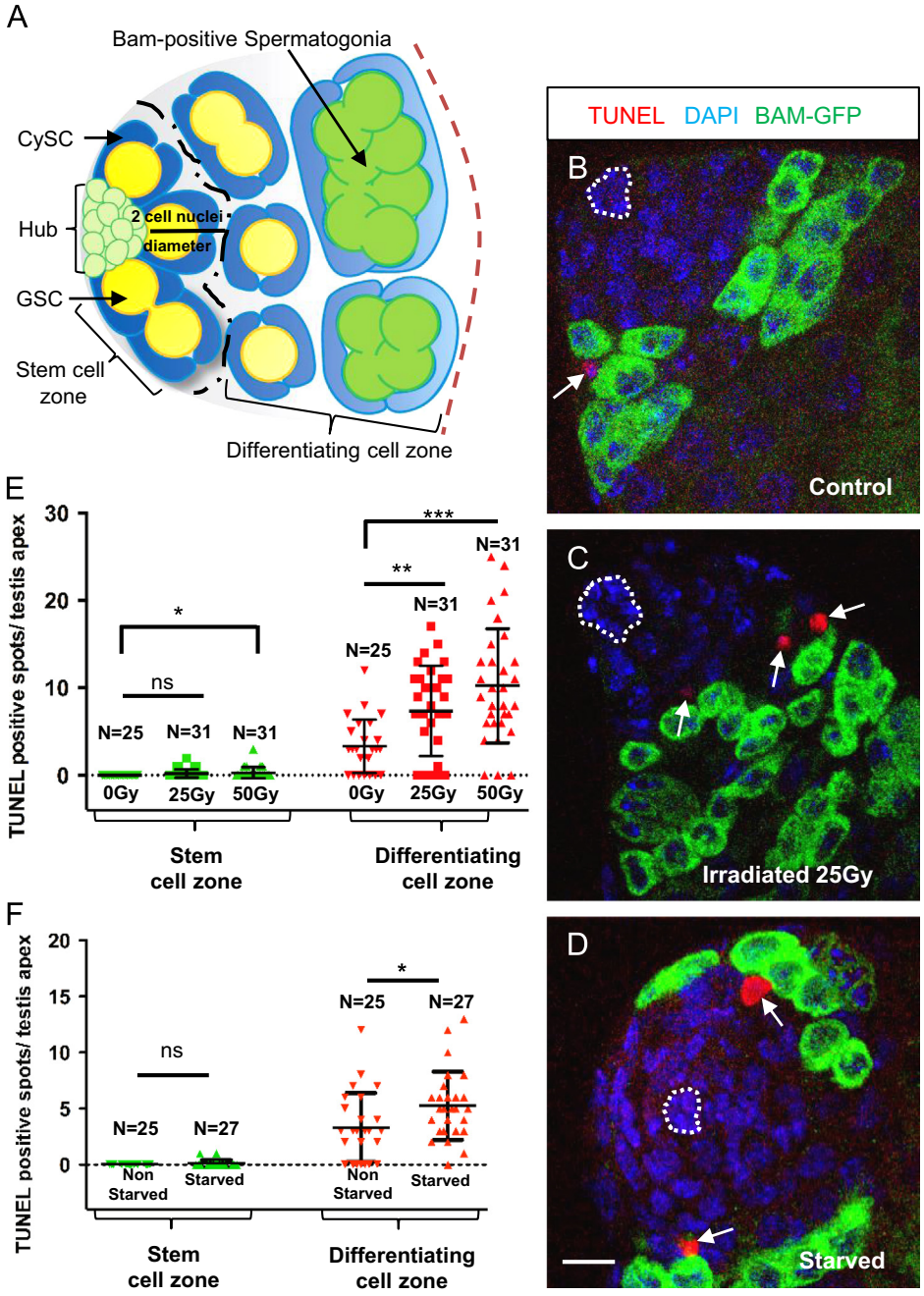

Fig. 1. Stem cells in the testis niche are more resistant to radiation and starvation induced cell death than differentiating cells. (A) Illustration of the *Drosophila* adult testis niche. GSCs (yellow) and CySCs (dark blue) are adjacent to the hub at the testis apex. GSCs divide to give rise to gonialblasts, which continue to divide, forming spermatogonial cysts. CySCs divide to form cyst cells, which envelop gonialblasts and their progeny. TUNEL-positive cells were quantified in two adjacent regions of the testis (1), A "Stem cell zone" comprised of the first two tiers of cells adjacent to the hub which includes all stem cells (GSCs and CySCs) and an occasional gonialblast and/or early cyst cell. (2), A "Differentiating cell zone", which extends from the boundary of the stem cell zone through the Bam-GFP positive spermatogonial cysts (green). (B, C, D) Confocal sections of testes expressing Bam-GFP fusion protein and immuno-stained with anti-GFP antisera (Bam expression pattern-spermatogonial cysts, green), TUNEL (apoptotic cells, red) and DAPI (DNA, blue) under control conditions (B), after 3 h of exposure to 25 Gy γ radiation (C) and after 19.5 h of protein-starvation (D). TUNEL-positive spots (arrows) are typically seen within and adjacent to Bam-positive spermatogonial cysts (C, D). Scale bar 10 μm. (E, F) Scatter plots showing the number of TUNEL-positive spots per testis apex in the stem cell and differentiating cell zones. (E) Upon 25 Gy of irradiation, there is no significant difference in TUNEL-positive spots in stem cell zone compared to un-irradiated controls. In contrast, 50 Gy of IR causes significantly more stem cells to die. In the differentiating cell zone, significantly more cells die under both 25 Gy and 50 Gy doses compared to un-irradiated controls. ($^{*}p < 0.05$), ($^{**}p < 0.01$). 2-tailed Kruskal–Wallis test with multiple comparisons. Bars indicate mean and S.D. (F) There is no significant difference in TUNEL-positive spots in the stem cell zone after starvation. There is a significant increase in TUNEL-positive spots in differentiating cell zone after starvation. ($^{*}p < 0.05$). 2-tailed Mann–Whitney test.

effector caspases, which triggers the cell death cascade (Fuchs and Steller, 2011).

Apoptosis is tightly regulated, and highly conserved anti-apoptotic proteins serve to block this typically irreversible process. This includes the inhibitor of apoptosis (IAP) and the B cell lymphoma 2 (Bcl-2) families of proteins, which inhibit caspase activation, thereby preventing their unwanted activity in the absence of death inducing stimuli (Fuchs and Steller, 2011; Ryoo and Baehrecke, 2010). Anti-apoptotic proteins are widely expressed and required, and increased expression of anti-apoptotic proteins in cells can confer resistance to apoptotic stimuli such as ionizing radiation-induced DNA damage (Liu et al., 2014). Indeed, it has been observed that radio-resistant stem cells such as HSCs have an enhanced expression of Bcl-2 (an anti-apoptotic member of the Bcl-2 family) compared to their daughters as studied in cell culture assays (Mohrin et al., 2010). In contrast, radio-sensitive ISCs have reduced expression of Bcl-2 when compared to radio-resistant, Bcl-2 enriched, colon stem cells as observed by immunohistochemistry for Bcl-2 in the murine small intestine (Liu et al., 2014; Merritt et al., 1995). However, not much is known about why stem cells in certain tissues have enhanced expression of anti-apoptotic proteins. There has been some recent evidence that Sox2, a transcription factor responsible for neural stem cell (NSC) self-renewal, also regulates expression of the IAP family member Survivin, which prevents NSC apoptosis under normal laboratory conditions (Feng et al., 2013). These data suggest that signals from the stem cell niche may cause an up-regulation of anti-apoptotic proteins specifically in stem cells, thereby making them cell death resistant.

Owing to its simplistic arrangement and the abundance of tools for genetic manipulations, the *Drosophila* testis niche is a useful model system to study the relation of stem cell survival to niche signaling. The *Drosophila* stem cell niche resides in the testis apex and consists of a cluster of quiescent niche cells called the hub (Fig. 1A). Two types of stem cells, the germline stem cells (GSCs) and the somatic cyst stem cells (CySCs), surround and adhere to the hub (Fig. 1A) (de Cuevas and Matunis, 2011; Matunis et al., 2012). The GSCs divide to give rise to differentiating daughter cells called gonialblasts, which divide to produce 2, 4, 8 and 16 cell interconnected spermatogonial cysts (Fig. 1A). The spermatogonia further differentiate into spermatocytes, which undergo meiosis to give rise to 64 sperm. The 4 and 8 cell spermatogonial clusters have high levels of the differentiating factor Bag of Marbles (Bam) when compared to GSCs and early daughters (Fig. 1A). Two CySCs envelop each GSC, and are required for the survival and proper differentiation of the germ cells. The nuclei of the CySCs are displaced from the hub relative to the nuclei of the GSCs. The CySCs divide to produce daughter cells called cyst cells. These cyst cells no longer divide, but instead elongate and differentiate as they encase the differentiating germ cells.

The hub produces various niche signals that are necessary for stem cell maintenance (de Cuevas and Matunis, 2011). One such signal is the ligand Unpaired (Upd), which binds to its receptor in both the GSCs and CySCs, activating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway in these cells (Kiger et al., 2001; Stine and Matunis, 2013; Tulina and Matunis, 2001). Activated STAT leads to the transcriptional up-regulation of genes within the stem cells that are important for their maintenance, while differentiating cells are located away from the hub and presumably receive less of the Upd signal. GSCs and CySCs require JAK and its downstream target STAT to prevent their differentiation (Flaherty et al., 2010; Hombría and Brown, 2002; Kiger et al., 2001; Leatherman and Dinardo, 2008; Ma et al., 2014; Stine et al., 2014; Tulina and Matunis, 2001). However, whether niche signals promote stem cell viability in this tissue has not been investigated.

Previous studies of cell death in the adult *Drosophila* testis have focused on dying differentiating germ cells (spermatogonia), which are readily detected in both *Drosophila* and mammalian testes. On average, 20–30% of differentiating spermatogonia die under normal laboratory conditions in every fly testis, even in testes from young adult males (Yacobi-Sharon et al., 2013); a similar percentage of spermatogonia also undergo cell death spontaneously in rodent testes (Allan et al., 1992). In both organisms, the type of germ cell death differs from canonical apoptosis as it includes morphological characteristics of both necrosis and apoptosis (Allan et al., 1992; Yacobi-Sharon et al., 2013). This includes cellular acidification and reactive oxygen species accumulation as seen in necrosis, as well as cellular shrinkage and chromatin condensation, which are characteristic of apoptosis (Yacobi-Sharon et al., 2013). Studies of stem cell death in both rodents and fly testes have suggested that the GSCs are more resistant to stress-induced death compared to their differentiating progeny (Dym and Clermont, 1970; Erickson, 1976; Ishii et al., 2014; Welshons and Russel, 1957). However, it is unclear how the GSCs are able to survive stress conditions which are lethal for their differentiating progeny. Here we investigate the occurrence of cell death within the GSCs and CySCs under normal laboratory conditions and stress-induced conditions, and determine whether niche signals and anti-apoptotic genes (IAPs) play a role in stem cell survival within the *Drosophila* testis niche.

Results

Stem cells are resistant to normal and stress-induced cell death

To determine whether stem cells, like spermatogonia, frequently undergo cell death under normal laboratory conditions, we sought to quantify and compare the number of dying stem cells and differentiating cells within the adult *Drosophila* testis. Dying cells in this tissue are identifiable through terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining; however, since TUNEL-positive cells are in advanced stages of dying (Gavrieli et al., 1992), they typically have lost expression of cell-type specific antigens (Darzynkiewicz et al., 1997). Thus, it is not straightforward to establish the identity of TUNEL-positive cells in many cases. Therefore, we focused our efforts on two zones based on testis morphology: the stem cell zone and the differentiating cell zone. A DNA counterstain was used to identify all nuclei, and the stem cell zone included all nuclei within the first two tiers from the hub (Fig. 1A). Since TUNEL-positive GSCs and CySCs are indistinguishable, but nuclei from the latter are displaced slightly further from the hub, selecting two tiers ensured the inclusion of all stem cells. It also meant that gonialblasts and early cyst cells were occasionally included in the stem cell zone, but this did not affect our results since TUNEL-positive cells were not detected within the stem cell zone under normal laboratory conditions ($N=25$ testes) (Fig. 1E and F). In comparison, dying germline cysts were readily detected in the differentiating cell zone, which included all nuclei immediately proximal to the stem cell zone to the end of the region of the testis containing Bam-positive spermatogonia (8–16 cell spermatogonial stage, Fig. 1A). On average, 3.32 TUNEL-positive spots per testis were seen in the differentiating cell zone in controls ($N=25$ testes) (Fig.1E and F). Since each testis in this genetic background contains an average of 16 spermatogonial cysts (Sheng et al., 2009), we estimate that 20.75% of the spermatogonial cysts are dying, which is consistent with the previously published estimate of 20% (Yacobi-Sharon et al., 2013). If GSCs die at a similar rate, we would expect to see 2 TUNEL-positive spots per testis within the stem cell zone since each testis in this genotype contains an average of 10 GSCs. However, we did not observe any dying cells in the stem cell zone in this genotype ($N=25$ testes) (Fig. 1E and F), which is a statistically

significant difference ($p=0.0001$, Chi-square test). Dying cells were never seen in the stem cell zone when flies were kept under standard laboratory conditions in all genotypes examined in this study ($N=177$ testes). Similarily, if CySCs were dying, we should have seen an even higher number of TUNEL-positive spots in the stem cell zone. Since we did not observe any dying cells in the stem cell zone, we conclude that stem cells in the adult *Drosophila* testis do not undergo cell death as often as their differentiating progeny when flies are kept under standard laboratory conditions.

The lack of dying stem cells in testes from well-fed young males suggested that stem cells are more resistant to death than spermatogonia. To pursue this further, we next asked whether stem cells and their daughters could differentially survive various environmental stresses. Since adult stem cells in some tissues are resistant to γ-irradiation (IR), which causes DNA double stranded breaks (DSBs) that result in apoptosis if unrepaired (Roos and Kaina, 2006), we chose to characterize the effects of IR on cells within the testis niche. Historical data suggested that stem cells but not differentiating cells survive 4000 Roentgens (or approximately 34 Gy) of ionizing radiation (Welshons and Russel, 1957). Therefore, we irradiated young adult males with 25 or 50 Gy of IR, and then examined the testis apex for the presence of TUNEL-positive spots. A significant increase in dying spermatogonial cysts (as measured by TUNEL-positive spots within the differentiating zone) was seen in testes from flies exposed to 25 Gy IR compared to un-irradiated controls (from 3.32 to 7.35 spots, $p<0.01$) (Fig. 1B, C and E), but no increase in cell death was observed in the stem cell zone (Fig. 1E). In contrast, exposure to 50 Gy of IR caused a significant increase in dying cells in both the stem cell ($p=0.048$) and differentiating cell ($p<0.001$) zones compared to un-irradiated controls (with 3% and 64% of the stem cells and spermatogonia, respectively, becoming TUNEL-positive) (Fig. 1E). We conclude that the increased resistance of stem cells in the *Drosophila* testis to cell death relative to their differentiating progeny applies to both normal laboratory conditions and irradiation conditions (25 Gy).

To confirm and extend these results we repeated our irradiation experiment (Fig. 1E) but analyzed testes with antisera against the cleaved (activated) effector caspase death caspase 1 (Dcp-1). In the ovary, this antibody labels nurse cells in dying egg chambers (Sarkissian et al., 2014). Since activation of Dcp-1 precedes fragmentation of DNA, marked by TUNEL, co-staining with anti-Dcp-1 and cell-specific antibodies can identify dying cells (Sarkissian et al., 2014). As expected, very few cleaved Dcp-1 positive cells were detected in wild-type control testes. Co-staining with anti-Traffic Jam (TJ) antisera to highlight CySC and cyst cell nuclei revealed an average of 0.1 CySCs and 0.65 cleaved Dcp-1 positive cyst cells per testis apex ($N=20$) (Supp. Fig. 1A and C). Since Dcp-1 is not required for the spontaneous spermatogonial cell death that occurs in flies kept under standard lab conditions (Yacobi-Sharon et al., 2013), we considered it likely that the male germline may not express this antigen. Indeed, cleaved Dcp-1 positive germ cells were never seen in control testes ($N=20$) (Supp. Fig. 1A). Upon 25 Gy of IR, as expected the number of cleaved Dcp-1 positive GSCs and CySCs was unchanged (an average of 0 GSCs and 0.2 CySCs per testis apex, $N=20$) (Supp. Fig. 1B and C) and the number of cleaved Dcp-1 positive cyst cells increased to an average of 1.75 per testis apex compared to 0.65 in un-irradiated controls (Supp. Fig. 1B and C). This is consistent with our results obtained with TUNEL staining and taken together, these results indicate that stem cells are more resistant to death under normal conditions and upon irradiation.

We next asked whether stem cells in the *Drosophila* testis are also resistant to milder, more physiologically relevant stresses, such as protein starvation. Protein starvation causes germline and somatic cells to die in the *Drosophila* ovary (Drummond-Barbosa and Spradling, 2001; Pritchett et al., 2009). Protein starvation also causes the number of GSCs to decrease due to differentiation in the testis, but its effects on cell death were not quantified (McLeod et al., 2010; Sheng and Matunis, 2011). Therefore, we placed flies on a protein starvation diet for 19.5 h, and then assayed dying cells in the testis using TUNEL labeling. This dietary change caused a significant increase in cell death in the differentiating cell zone but not in the stem cell zone compared to un-starved controls (Fig. 1B, D and F). We conclude that the ability of stem cells in the *Drosophila* testis to resist death better than their differentiating progeny is seen both under normal laboratory conditions, under extreme stress such as IR, and under more moderate stress such as temporary protein starvation.

JAK–STAT signaling by niche cells causes DIAP1 enrichment in stem cells and their immediate daughters

To understand why stem cells in the *Drosophila* testis are more resistant to cell death than their differentiating progeny, we focused on the anti-apoptotic factor *Drosophila* inhibitor of apoptosis 1 (DIAP1). DIAP1, encoded by the essential gene *thread* (Hay et al., 1995), is the best characterized IAP (Inhibitor of Apoptosis) family protein found in flies. DIAP1 binds to and inhibits the initiator caspase Dronc and the effector caspases DrICE and Dcp-1 (Meier et al. 2000b; Wang et al., 1999; Wilson et al., 2002), and blocks apoptosis during normal development and after irradiation (Betz et al., 2008; Meier et al. 2000a; Ou et al., 2003). Expression of pro-apoptotic proteins like Reaper, Grim or Hid (Head involution defective) can inhibit DIAP1 and cause cell death (Chai et al., 2003; Fuchs and Steller, 2011). Both GSCs and CySCs can be forced to die by overexpression of Hid and Grim respectively (Hetie et al., 2014; Lim and Fuller, 2012), Greenspan and Matunis (unpublished data). Immunostaining young adult testes with anti-DIAP1 antisera revealed that DIAP1 is weakly expressed throughout the testis and is enriched in both GSCs and the CySCs relative to their differentiating progeny (Fig. 2A and A′).

The enrichment of DIAP1 in the stem cells of the *Drosophila* testis suggests that niche signals may be regulating *diap1* expression. JAK–STAT signaling is a major niche signaling pathway in the *Drosophila* testis and the single *Drosophila* STAT, STAT92E, is locally activated within GSCs and CySCs to promote their maintenance (Brawley and Matunis, 2004; Kiger et al., 2001; Leatherman and Dinardo, 2008). *diap1* is transcriptionally up-regulated by JAK–STAT signaling in the *Drosophila* wing imaginal disk (Betz et al., 2008), suggesting it could be a STAT target in the testis apex as well. To determine if *diap1* is transcriptionally regulated by STAT in GSCs and CySCs, we used established procedures to either reduce or increase JAK–STAT signaling in the testis (Issigonis and Matunis, 2012; Issigonis et al., 2009; Stine et al., 2014), then assayed for changes in DIAP1 levels soon thereafter, prior to stem cell loss. To reduce JAK–STAT signaling, we shifted adult flies carrying a temperature sensitive allele of *STAT92E* to non-permissive temperature for 24 h. We observed a substantial decrease in DIAP1 levels in all cells within the testis apex, especially cells closest to the hub (Fig. 2B and B′). Quantification of DIAP1 expression levels within the stem cells revealed a significant decrease in protein expression when compared to controls (Fig. 2D). To increase JAK–STAT signaling, we ectopically expressed the JAK–STAT ligand *unpaired* briefly throughout the testis. This caused an up-regulation of DIAP1 protein in all cells within the testis apex, including stem cells and their differentiating progeny (Fig. 2C and C′). Quantification of DIAP1 levels specifically in GSCs and CySCs from these testes revealed a statistically significant increase in DIAP1 expression levels compared to control testes (Fig. 2E). Together, these results indicate that DIAP1 is a direct or indirect target of JAK–STAT signaling in the *Drosophila* testis, and support the hypothesis that the increased expression of DIAP1

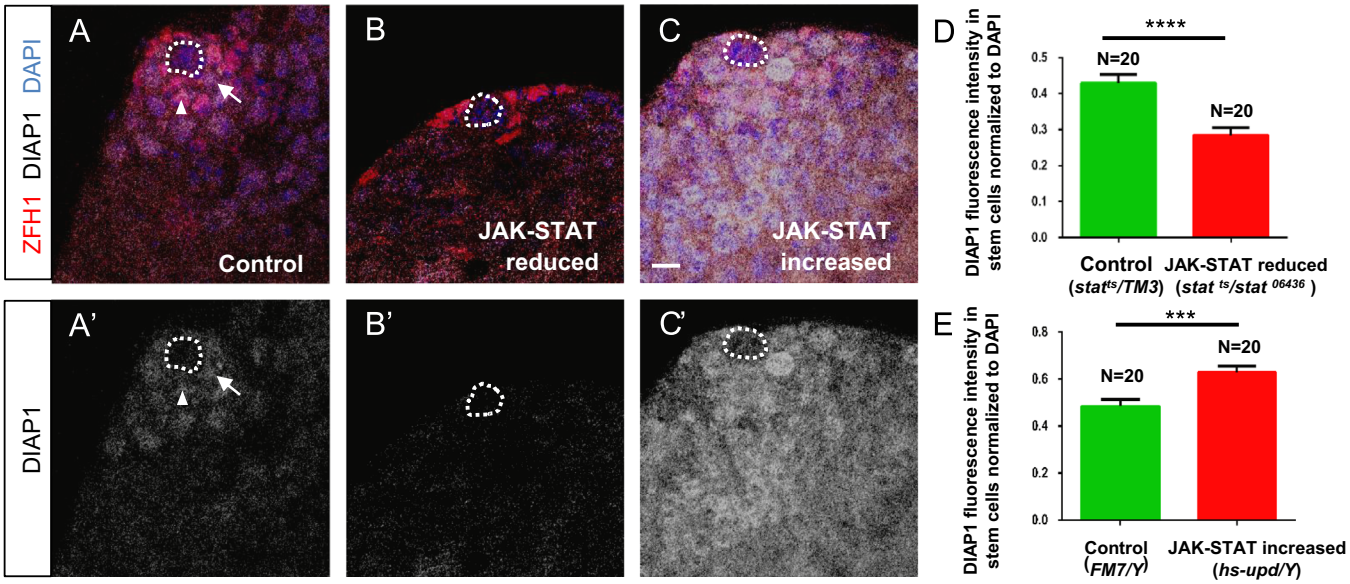

Fig. 2. DIAP1 is enriched in the stem cells and is a target of JAK–STAT signaling.
(A–C) Confocal sections through the apex of testes stained with anti-DIAP1 (white), anti-Zfh1 (CySCs and cyst cells, red), and DAPI (DNA, blue). (A′–C′) DIAP1 channel alone. In control testes, DIAP1 protein is enriched around the hub (white outline) and co-localizes with CySCs (Zfh1 positive cells near the hub; one indicated, arrowhead) and GSCs (Zfh1- negative cells contacting the hub; one indicated, arrow). (B) DIAP1 levels are reduced in testes with reduced JAK–STAT signaling (genotype: $stat^{F}$/$stat^{06436}$) after 24 h at restrictive temperature (see Methods). (C) DIAP1 levels are increased in both stem cell populations and in differentiating cells in testes with increased JAK–STAT signaling (*hs-upd* testes after 45 min of induction). (D, E) Bar graph showing quantification of DIAP1 levels in stem cells normalized to DAPI levels in the same cells in control (green) and experimental (red) flies when JAK–STAT is reduced (D) or up-regulated (E) (***$p < 0.001$), (****$p < 0.0001$). 2-tailed Mann–Whitney test. Error Bars indicate mean and S.E.M.

within the stem cells compared to their differentiating progeny seen in wild-type control testes is due to niche signaling.

DIAP1 is necessary for stem cell survival

Because the stem cells of the *Drosophila* testis are enriched in DIAP1 and are highly stress-resistant, we next wanted to investigate DIAP1 function in these cells. DIAP1 is best characterized as an anti-apoptotic protein (Hay et al., 1995), but also has non-apoptotic roles, including regulating border cell migration during oogenesis (Geisbrecht and Montell, 2004). We used two independent approaches to determine the requirement for DIAP1 in GSCs and CySCs: 1) mosaic analysis of *thread* (or DIAP1) null GSC and CySC clones, and 2) global RNAi-mediated knockdown of DIAP1. In the first approach, we used two loss-of-function alleles of *thread* to create negatively marked DIAP1 null CySC and GSC clones (see Methods). By tracking clones through the absence of GFP, we found that by 2 days after clone induction (ACI) there were already significantly fewer DIAP1 null GSCs and CySCs compared to control wild-type clones (Fig. 3A–C, Table 1). This suggested that the majority of DIAP1 null stem cells are lost rapidly, since most were no longer visible by 2 days ACI, which is the earliest time point the marking system is visible (Corish and Tyler-Smith, 1999). The loss of DIAP1 null stem cells could be due to their differentiation or death. If stem cell clones are lost by differentiation we would expect to see a robust number of differentiating clones 2 days ACI. Compared to wild type clones, very few differentiating germline and somatic cells lacking DIAP1 were detected (Table 1), suggesting that the DIAP1 null GSC and CySC clones are rapidly dying, rather than entering the differentiation pathway. Consistent with this observation, DIAP1 null clones are lost very rapidly in the larval imaginal disk, the adult eye, and the ovary due to cell death (Hay et al., 1995; Ryoo et al., 2004), and DIAP1 ablation in spermatogonial cysts results in their death (Yacobi-Sharon et al., 2013). Therefore, we consider it most likely that DIAP1 null clones in the adult testis are rapidly lost due to cell death, rather than differentiation. We did notice that DIAP1 null GSCs were lost less rapidly than the DIAP1 null CySCs (Table 1), which suggests that they might contain other anti-apoptotic proteins apart from DIAP1. This possibility is consistent with the presence of alternative germ cell death pathways in spermatogonia (Yacobi-Sharon et al., 2013).

To confirm and extend our clonal analysis, we performed conditional global induction of DIAP1 RNAi to reduce DIAP1 levels in adult testes. Induction of DIAP1 RNAi (under control of the Heat shock promoter) resulted in a reduction of DIAP1 protein levels throughout the entire testis including the niche region (Supp. Fig. 2A–D). Prior to RNAi induction, very few dying cells were detected in the stem cell zone in both control and experimental testes. At 3 h post RNAi induction, the number of TUNEL-positive cells within the stem cell zone increased significantly in testes lacking DIAP1 relative to controls (from 0.18 in un-induced DIAP1 RNAi control to 1.36 dying cells per testis, Fig. 3D–F) (the few dying stem cells in un-induced DIAP1 RNAi control could be due to background induction of DIAP1 RNAi at 25 °C). Importantly, this increase in TUNEL-positive cells is not due to experimental conditions: testes expressing a control RNAi (against GFP) did not have a significant increase in number of TUNEL-positive cells upon heat-shock (Fig. 3F). Finally, expressing DIAP1 RNAi specifically in the CySC lineage significantly decreased the number of CySCs (Supp. Fig. 3A). The significant increase in stem cell death upon DIAP1 RNAi induction, coupled with the rapid loss of DIAP1 null stem cell clones, supports the conclusion that DIAP1 is required for survival of GSCs and CySCs in the testis niche.

Decreased JAK–STAT signaling makes the stem cells susceptible to stress-induced death

Reducing STAT levels (by shifting stat temperature-sensitive flies to the non-permissive temperature) causes a decrease in DIAP1 levels (Fig. 2), but stem cells in these testes undergo differentiation, rather than cell death (Brawley and Matunis, 2004),

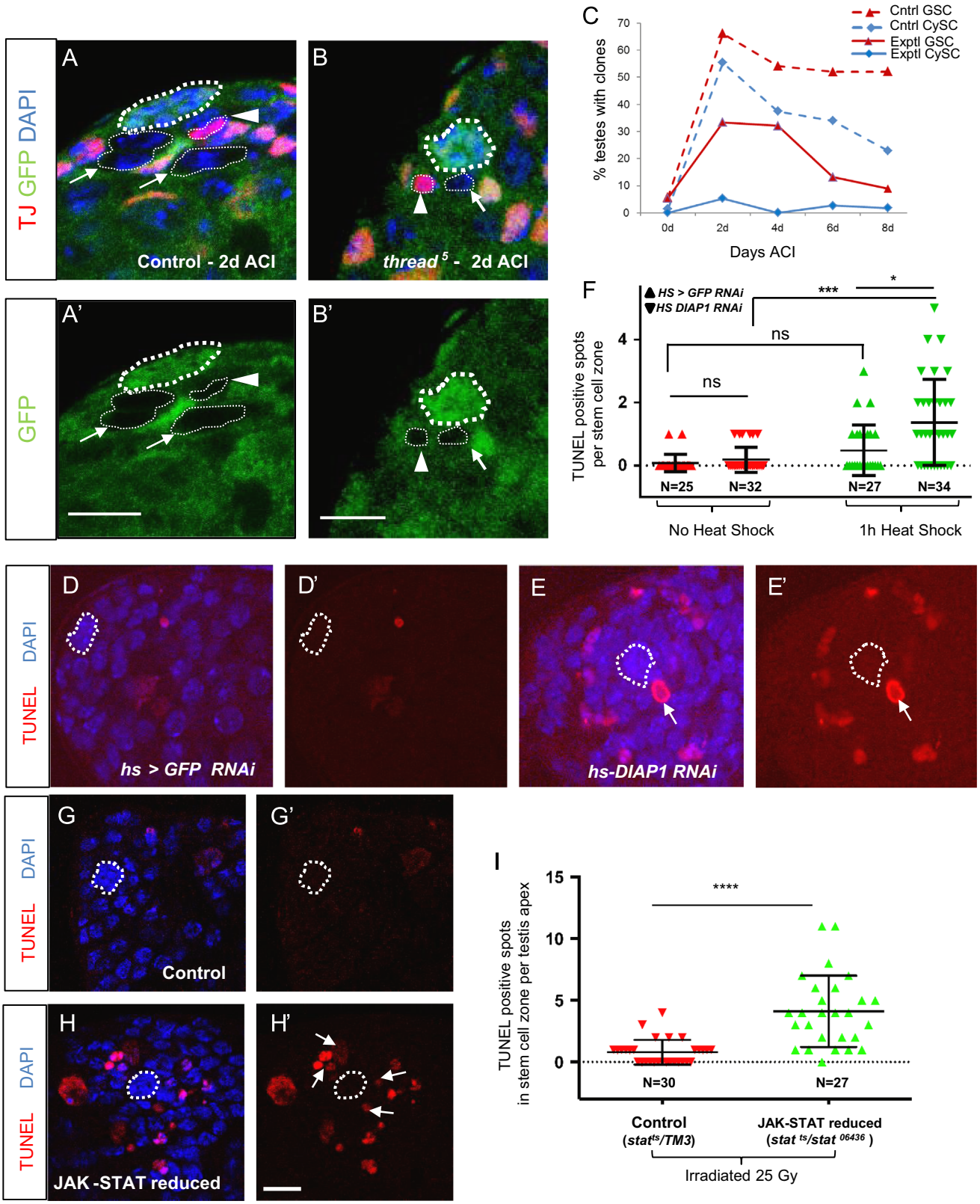

Fig. 3. DIAP1 is required for stem cell survival. (A, B) Confocal sections through the testis apex show control (A) or DIAP1 null (*thread*^{5}, B) clones 2 days after clone induction (ACI). Hubs are outlined in white (thick line). Negatively marked clones, identified by the absence of GFP (green) staining, are outlined in white (thin line). Clones positive for Tj (red) are CySC lineage clones (arrowheads), and those negative for Tj are germ line clones (arrows). Both control (A, A′) and DIAP1 null (B, B′) clones can be seen next to the hub. (C) Line diagram showing the percentage of testes with DIAP1 null (*thread*^{5}, Exptl) or control (Cntrl) GSC or CySC clones after clone induction. Very few *thread*^{5} CySC clones (blue solid line) and *thread*^{5} GSC clones (red solid line) are seen compared to control CySC (blue dashed line) or GSC (red dashed line) clones. (D–H) Confocal sections through the apex of testes stained with TUNEL (red) and DAPI (blue). Hubs are outlined in white. (D′–H′), TUNEL channel alone. (D, D′) TUNEL positive cells next to the hub were rarely seen in control testes after induction of GFP RNAi. (E, E′) After heat shock-mediated induction of DIAP1-RNAi (E), TUNEL positive cells were seen adjacent to the hub (arrow in E, E′). (F) Scatter plot showing quantification of data from D and E. The number of dying cells in the stem cell zone (see Methods) increased significantly upon induction of DIAP1 RNAi compared to induction of GFP RNAi in controls ($^*p < 0.05$). No significant difference in cell death was observed without induction of RNAi or with induction of GFP RNAi. Statistical test used is Kruskal–Wallis test of multiple comparisons. (G, G′) Irradiation of control testes (genotype- *stat*^{F}/*TM3*, after 24 h at restrictive temperature) at 25 Gy did not result in TUNEL positive cells next to the hub (stem cell zone). (H, H′) Irradiation of testes with reduced JAK–STAT signaling (genotype: *stat*^{F}/*stat*^{06436}, after 24 h at restrictive temperature) at 25 Gy resulted in increased number of TUNEL positive cells next to the hub (arrows in G, G′) (stem cell zone). Scale bars 10 μm. (I) Scatter plot shows quantification of data from G and H. The number of TUNEL positive cells in the stem cell zone increased significantly upon irradiation (25 Gy) after reduction of JAK–STAT signaling compared to irradiated control testes ($^{****}p < 0.0001$). 2 tailed Mann–Whitney test. Bars (F, I) indicate mean and S.D.

Table 1
DIAP1 is required for maintenance of both GSCs and CySCs.

Days ACI	*thread* Allele	Percentage of testes with clones			
		GSC clones	CySC clones	Spermatogonial clones	Cyst cell clones
No HS control	Wild type	6.3% (6/95)	3.1% (3/95)	9.4% (9/95)	3.1% (3/95)
	thread^4	5.3% (1/19)	5.3% (1/19)	5.2% (1/19)	5.2% (1/19)
	thread^5	5.4% (3/55)	0% (0/55)	3.6% (6/55)	3.6% (2/55)
	p-Value	n.s.	n.s.		
2	Wild type	65.3% (62/95)	50.5% (48/95)	77.8% (74/95)	45.2% (43/95)
	thread^4	26.1% (6/23)	21.7% (5/23)	8.7% (2/23)	8.7% (2/23)
	thread^5	33.8% (25/74)	5.3% (4/74)	8.1% (6/74)	6.7% (5/74)
	p-Value	$p < 0.0001$	$p < 0.0001$		
4	Wild type	59.3% (32/53)	35.2% (19/53)	64.1% (34/53)	39.6% (21/53)
	thread^4	12.5% (2/16)	0% (0/16)	18.7% (3/16)	6.2% (1/16)
	thread^5	15.4% (4/26)	0% (0/26)	3.8% (1/26)	7.7% (2/26)
	p-Value	$p=0.001$	$p < 0.0001$		
6	Wild type	52.0% (26/51)	34.0% (17/51)	55% (28/51)	53% (27/51)
	thread^5	13.5% (5/37)	2.6% (1/37)	5.4% (2/37)	8% (3/37)
	p-Value	$p=0.0002$	$p=0.0003$		
8	Wild type	52.1% (25/47)	22.9% (11/47)	55% (26/47)	34% (16/47)
	thread^5	8.9% (5/55)	1.8% (1/55)	3.6% (2/55)	1.8% (1/55)
	p-Value	$p < 0.0001$	$p=0.0011$		
10	Wild type	47.4% (9/19)	15.8% (3/19)	52.6% (10/19)	10.5% (2/19)
	thread^4	4% (1/23)	0% (0/23)	4% (1/23)	0% (0/23)
	p-Value	$p=0.0023$	n.s.		

Testes with one or more clones have been quantified both as a fraction of total number of testes and as percentages (in brackets). *p*-Values based on Chi Square and Fisher's exact test. *p*-Values are based on comparisons between the percent testes with wild-type GSC or CySC clones and percent testes with DIAP1 null GSC or CySC clones (including both *thread*^{4} and *thread*^{5} clones). HS= Heat shock, ACI= After clone induction.

suggesting that the low levels of DIAP1 that remain after reduction of STAT are sufficient to protect stem cells from death under physiological conditions. However, it also suggested that the reduced levels of DIAP1 would render these cells more sensitive to stress-induced cell death. To test this hypothesis, we irradiated flies with decreased STAT levels (see Methods) with 25 Gy IR and then used TUNEL staining to quantify the number of dying cells in the testis apex. We found significantly more TUNEL positive cells in the stem cell zone in the irradiated testes with decreased JAK–STAT signaling (4.11 dying cells per testis apex, $N=27$ testes) compared to irradiated controls with normal JAK–STAT signaling (0.8 dying cells per testis apex, $N=30$ testes; Fig. 3G–I). We conclude that decreasing JAK–STAT signaling sensitizes the stem cells to stress-induced cell death, likely because of reduction in DIAP1 levels.

Up-regulation of DIAP1 in differentiating somatic cells, but not germ cells, rescues stress-induced spermatogonial death

Stem cells in the *Drosophila* testis resist cell death better than their differentiating progeny (Fig. 1), have an enrichment of DIAP1 (Fig. 2), and require this anti-apoptotic protein for their survival (Fig. 3). However, since dying stem cells are never detected in flies kept under standard laboratory conditions, we cannot tell if the higher levels of DIAP1 within stem cells are sufficient to confer their survival advantages. Ideally we could reduce the DIAP1 levels in the stem cells to match those seen in spermatogonia, and then ask if stem cells die more often, but this is not technically possible: even moderate RNAi knockdown of DIAP1 causes stem cells to die, and flies heterozygous for the null allele *thread*^{4} did not show a noticeable increase in stem cell death in the testis (data not shown). Similarly, *thread* null heterozygotes do not show any increase in neuronal or eye cell death (Choi et al., 2006; Hawkins et al., 2000; Wang et al., 1999). Thus, though indirect reduction of DIAP1 by decreasing JAK–STAT signaling rendered the stem cells susceptible to stress-induced death, the precise level of DIAP1 required for stem cell maintenance in the testis is not known. We do know, however, that ectopically up-regulating DIAP1 levels in GSCs or CySCs does not adversely affect their viability: we never saw dying GSCs or CySCs in testes where DIAP1 is overexpressed in GSCs or CySCs respectively (Figs. 4A–B′, D, D′) (data not shown). Furthermore, the Arama lab showed that up-regulating DIAP1 in differentiating germ cells is not sufficient to rescue these cells from death, a result that we have confirmed using both Nanos and Bam-Gal4 drivers (Fig. 4E, Supp. Fig. 4B–D). This is because, as mentioned above, normal spermatogonial death occurs by a combination of apoptosis and necrotic pathways and does not involve the canonical apoptotic pathway, which DIAP1 inhibits (Fuchs and Steller, 2011; Yacobi-Sharon et al., 2013). Therefore, we wondered if stress-induced cell death could differ from the non-canonical cell death that spermatogonia undergo under normal laboratory conditions (Yacobi-Sharon et al., 2013). If so, stress-induced cell death could be rescued by up-regulation of DIAP1 in the spermatogonia. To explore this possibility, we up-regulated DIAP1 in 4–16 cell spermatogonial clusters (using the Bam-Gal4 driver) and subjected the flies to stress (protein starvation and irradiation). We found that the spermatogonial cell death induced by protein starvation or irradiation is not rescued by up-regulation of DIAP1 (Supp. Fig. 4A–C). We conclude that up-regulating DIAP1 in the late spermatogonial cells is insufficient to autonomously rescue them from cell death that occurs under laboratory conditions, or under starvation-induced stress.

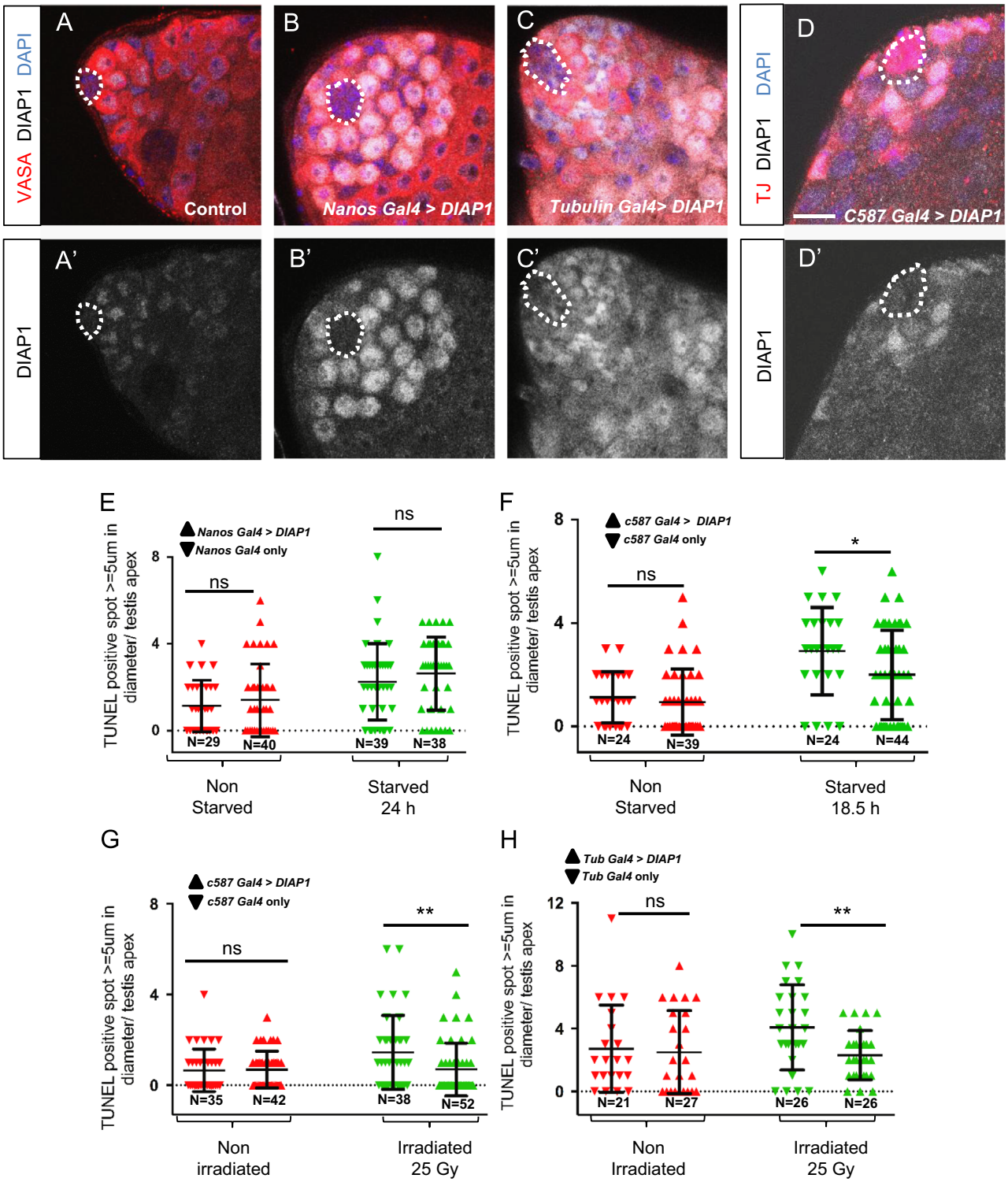

Fig. 4. DIAP1 over-expression in CySCs and their daughters rescues differentiating germline cells from stress induced cell death. (A–D) Confocal sections through the apex of testes stained with anti-DIAP1 (white), anti-Vasa (A–C, germ cells, red) or anti-Tj (D, somatic cells, red), and DAPI. (A′–D′) DIAP1 channel alone. Testes were imaged at the same gain to show different levels of DIAP1 protein in (A, A′) a control testis or in testes overexpressing DIAP1 in (B, B′) GSCs and their daughters using Nanos Gal4; (C, C′) in both germ cells and somatic cyst cells using Tubulin Gal4; (D, D′) in CySCs and their daughters using c587 Gal4. Hubs are outlined in white. Scale bar, 10 μm. (E–H) Scatter plots showing quantification of TUNEL-positive spermatogonial cell clusters (see the text) under normal conditions or under stress from protein starvation (E, F) or irradiation (G, H). Over expression of DIAP1 in GSCs and their daughters (E) under normal or protein starvation conditions does not significantly alter the number of TUNEL positive spermatogonial cell clusters compared to controls without DIAP1 over expression. However, there is a decrease in TUNEL positive spermatogonial cell clusters under both protein starvation and irradiation conditions in testes over expressing DIAP1 in CySCs and their daughters (F, G). Simultaneous over expression of DIAP1 in both germ cells and somatic cyst cells also results in a decrease in TUNEL positive spermatogonial cell clusters under irradiation conditions (H) ($^{*}p < 0.05$), ($^{**}p < 0.01$). 2-tailed Mann–Whitney test. In all graphs, bars indicate mean and S.D.; sample size for each treatment set indicated under the respective plot.

Since over-expressing DIAP1 directly in the germ cells was not sufficient to promote their survival under protein starvation conditions, we hypothesized that some spermatogonia may be dying as a secondary consequence of the death of their accompanying somatic cyst cells. Cyst cells envelop differentiating germ cells (Zoller and Schulz, 2012) and are vital for the survival and proper differentiation of the spermatogonia (Supp. Fig. 3B) (Hetie et al., 2014; Lim and Fuller, 2012; Zoller and Schulz, 2012). Up-regulating DIAP1 levels in cyst cells using the c587 Gal4 driver (Fig. 4D and D′) in flies reared under normal conditions had no effect on spermatogonial cell death, indicating that these germ cells are not likely dying due to cyst cell death (Fig. 4F and G controls). However, the additional dying spermatogonia that arise after protein starvation or irradiation are rescued by boosting DIAP1 levels in the CySC lineage: the average number of dying spermatogonial clusters in starved males decreased significantly from 2.92 to 1.97 upon overexpression of DIAP1 in cyst lineage cells (N= 24, 44) ($p < 0.05$) (Fig. 4F). Similarly, DIAP1 over-expression in the CySC lineage rescued the additional spermatogonial death induced by irradiation. The average number of dying spermatogonial clusters per testis apex in flies subjected to 25 Gy of γ-radiation (which kills spermatogonia and somatic cyst cells but not stem cells) (Fig. 1) decreased significantly from 1.34 to 0.69 upon over-expression of DIAP1 in CySC lineage cells ($p < 0.01$) (Fig. 4G). This is similar to the level of death seen in un-irradiated control flies without DIAP1 up-regulation (Fig. 4F and G). We conclude that expression of ectopic DIAP1 in the somatic cells non-autonomously rescues stress-induced spermatogonial cell death.

If overexpression of DIAP1 in CySC lineage cells but not in germ cells is sufficient to rescue stress-induced spermatogonial cell death, then overexpression of DIAP1 in both somatic and germ cells should phenocopy overexpression of DIAP1 in the CySC lineage alone. Therefore, we used a tubulin Gal4 driver to over-express DIAP1 in both the somatic and germline cells (White-Cooper, 2012). Consistent with published results, and our data in Fig. 4E–G, we did not observe any rescue in spermatogonial death in these flies prior to irradiation (Yacobi-Sharon et al., 2013) (Fig. 4H). After 25 Gy of irradiation, we found a significant decrease in the average number of dying spermatogonial clusters (from 4.2 to 2.3 per testis apex) ($p < 0.01$) upon overexpression of DIAP1 in germline and soma. Thus, ectopic DIAP1 rescues close to 50% of the spermatogonial clusters from IR induced death when it is expressed in either the germline and soma or the soma alone. Taken together, these results indicate that stress-mediated increase of spermatogonial cell death can be completely rescued non-autonomously by over-expression of DIAP1 in the somatic CySC lineage.

Cyst cells are more susceptible than germline cells to stress-induced cell death

Since we could fully rescue the stress-induced increase in spermatogonial cell death by over-expressing DIAP1 in somatic cyst cells but not in germ cells, we hypothesized that this increase in spermatogonial cell death may be secondary to the loss of the cyst cells. If so, we may be able to detect an increase in the number of dying cyst cells adjacent to living germ cell clusters under stress conditions. To identify dying cyst cell-living germ cell cluster pairs, we used the antibody against cleaved Dcp-1 protein, which marks dying somatic cells under normal and stress conditions (Supp. Fig. 1A and B), an antibody against Traffic jam to mark somatic cell nuclei, and expression of Bam-GFP fusion protein to mark spermatogonial cell clusters. We assumed that the germline cells which are expressing Bam-GFP are still alive. We counted the number of dying cyst cell-living germ cell pairs in testes from both irradiated (25 Gy) and un-irradiated controls. The number of these pairs increased significantly from an average of 0.6 to 1.1 per testis apex upon irradiation (N=20 testes) (Fig. 5 A and B). We hypothesize that the Dcp-1 positive cyst cells in these pairs are expressing activated effector caspase and are progressing through apoptosis while the enclosed germline cells are living. However, we cannot rule out the possibility that the enclosed germ cells are expressing cell death markers different from cleaved Dcp-1, since markers of early stages of germ cell death that can be visualized along with cell-specific protein expression have not yet been identified. Our data therefore suggest that cyst cells are more susceptible to stress-induced cell death than the enclosed germline cluster and start progressing towards cell death when the associated germline cluster is still living.

Altogether, our results support a model where niche signaling makes the stem cells resistant to stress-induced cell death. The differentiating cells lack the resistance to stress-induced cell death, with cyst cells being more susceptible to stress-induced cell death than their accompanying germ cell partners.

Discussion

Both germline and somatic stem cells in the adult *Drosophila* testis are more resistant to apoptosis-inducing stimuli than their differentiating daughters. Niche signaling causes enhanced expression of DIAP1 in both GSCs and CySCs, and the resistance to apoptosis is mediated, at least in part, by this enhanced expression.

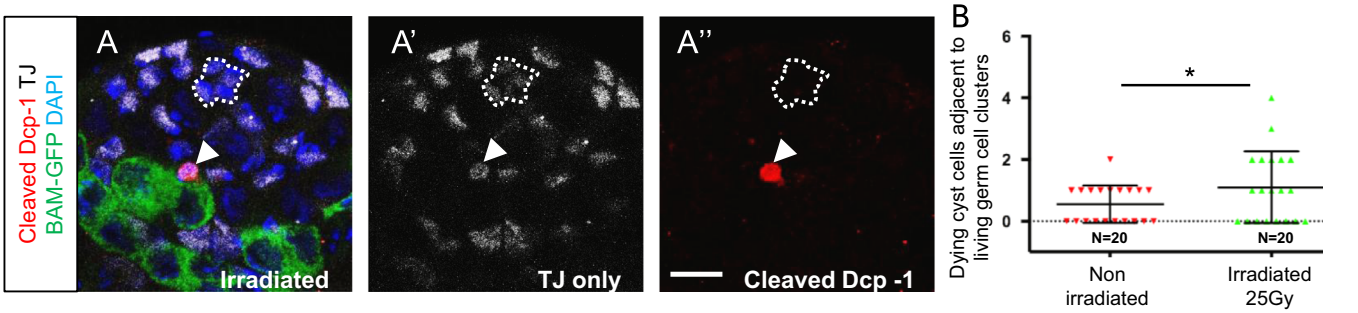

Fig. 5. Cyst cells die before the enclosed germ cell clusters under stress condition. (A–A″) Confocal section through the apex of a testis expressing Bam-GFP fusion protein and stained with anti-TJ (somatic cells, white, A′), anti- cleaved Dcp-1 (Death caspase-1: effector caspase activity, red, A″) or anti-GFP (Bam expression pattern: germ cell clusters, green), and DAPI. (A) A dying cyst cells (TJ positive) with cleaved Dcp-1 (white arrow) was seen adjacent to a living (cleaved Dcp-1 negative) germ cell cluster (Bam expression) after irradiation (25 Gy). Scale bar 10 μm. (B) Scatter plots showing quantification of dying cyst cell (TJ positive, cleaved Dcp-1 positive)-living germ cell cluster (Bam expression) pair in a spermatogonial cyst under control or irradiation conditions. Dying cyst cell-living germ cell cluster pairs are seen in control as well as irradiated testes. Significantly more dying cyst cell-living germ cell cluster pairs are seen under irradiation conditions (*$p < 0.05$). 1-tailed Student's T-test. In all graphs, bars indicate mean and S.D., sample size for each treatment set indicated under the respective plot.

Niche signaling can enhance survival of stem cells

Stem cells in various mammalian adult tissues have enhanced expression of anti-apoptotic proteins such as Bcl2 and IAPs. However, little is known about how these proteins are regulated, or whether signals from the niche play a role in their up-regulation. Here, we find that niche signaling can play a direct role in promoting survival of stem cells by up-regulating the expression of pro-survival genes. We show that the anti-apoptotic protein DIAP1 is a transcriptional target of JAK–STAT signaling in both the CySCs and the GSCs in the testis. STAT binds directly to the DIAP1 promoter region, and this binding is sufficient to cause up-regulation of DIAP1 in 3rd instar larval wing discs (Betz et al., 2008). Therefore, it is likely that STAT acts directly on DIAP1 in testis stem cells, but future experiments are needed to determine this. STAT has also been shown to up-regulate anti-apoptotic proteins in cycling adult satellite stem cells in mammalian muscle (Golding et al., 2007), but whether STAT activation is a consequence of niche signaling in this tissue remains to be determined.

Since DIAP1 is a STAT target, and loss of DIAP1 leads to cell death, one might expect STAT null testes to lose stem cells by cell death. However, STAT null testes appear to lose stem cells by differentiation and not cell death (Brawley and Matunis, 2004). This could be due to the fact that STAT is a transcription factor regulating the expression pattern of multiple genes. Some of these genes regulate stem cell renewal, and it is possible that reduced expression of these genes causes stem cell differentiation to occur before DIAP1 expression is reduced enough to cause cell death. Alternatively, DIAP1 may also be regulated by another pathway in the testis and its levels may not be affected at the same rate as some of the more direct targets of STAT (such as the targets involved in renewal). Consistent with this idea, DIAP1 levels are not reduced completely upon reduction of STAT (see methods for STAT reduction procedures). Although the decrease in STAT levels does not induce cell death in the stem cells, similar decrease makes them sensitive to stress-induced cell death. Identifying other regulators of DIAP1, which may be involved in DIAP1 up-regulation in stem cells, could help to distinguish between these possibilities. Other targets of niche signaling may also have an anti-apoptotic roles in stem cells. Possible candidates include the IAP family member *Drosophila* inhibitor of apoptosis 2 (DIAP2). According to published RNA-seq data there seems to be an enrichment of DIAP2 in testes (Chintapalli et al., 2007). However, although DIAP2 is an IAP family member, so far it has been thought to be primarily important for innate immunity, rather than cell survival (Gesellchen et al., 2005; Huh et al., 2007; Leulier et al., 2006).

Cyst cells play a role in germ cell survival

Cyst cells are known to play a role in germ cell survival (Hetie et al., 2014; Zoller and Schulz, 2012). Our data shows that cyst cells can be responsible for death of germ cell clusters due to their susceptibility to stress-induced cell death. The susceptibility of cyst cells to stress conditions could be one of the reasons why stress-induced spermatogonial cell death can be rescued non-autonomously by DIAP1 overexpression in cyst cells. Similar non-autonomous regulation of germline cell death by somatic cell has been demonstrated in adult *Drosophila* ovary where induction of cell death in follicle cells (somatic lineage) leads to nurse cell death (germ cell lineage) (Chao and Nagoshi, 1999). It may be interesting to analyze why cyst cells are more susceptible to stress-induced death than their enclosed germ cells as well as what kind of cross-talk goes between the cyst cells and their enclosed germ cell clusters that leads to germ cell death once cyst cells are lost. Our data, therefore, supports and extends the published work further, providing another role of cyst cells in survival of the enclosed germ cell cluster. However, we must note that, although DIAP1 over expression in cyst cells can prevent stress-induced spermatogonial cell death, it does not rescue normal spermatogonial death.

GSCs in the testis are radio-resistant

The enhanced capability of male GSCs to survive radiation-induced cell death has been well documented in *Drosophila* testis (Welshons and Russel, 1957). Classic studies in *Drosophila* showed that following irradiation, males are fertile for a while then enter a period of temporary sterility, before recovering fertility (Welshons and Russel, 1957). The period of temporary sterility is proportional to the intensity of radiation (Welshons and Russel, 1957). Welshons and Russel studied the morphological characteristics of the testis after irradiation and concluded that the testis looked completely normal 15 days post-irradiation with a 4000 R (35 Gy) X ray dosage (Welshons and Russel, 1957). Microscopic observation of the irradiated testes indicated that the spermatogonia are susceptible to radiation and undergo apoptosis while the spermatocytes were relatively more resistant to radiation (Welshons and Russel, 1957). Although the fate of the stem cells in these studies was not determined, the full restoration of fertility after a temporary lapse suggests that the stem cells were resistant to radiation. Our work analyzes the effect of irradiation on stem cells and explains the phenomenon of temporary sterility. The stem cells are not as susceptible as the spermatogonia to radiation-induced apoptosis, and survive, providing a source of new germ cells post-irradiation. Thus, our work indicate that temporary sterility is induced due to the elimination of spematogonia upon irradiation, and fertility is restored when the stem cells replenish the spermatogonial pool. The ability of male GSCs to resist death extends to mammalian systems including rat and mouse testis, where GSCs can also divide to restore the fertility of irradiated mice and rats (Dym and Clermont, 1970; Erickson, 1976; Withers et al., 1974). Our data agrees with the mammalian data and suggests that niche signals may make GSCs more resistant to stress conditions than their differentiating progeny across a diverse set of organisms.

Implications in regulation of cancer stem cells

Up-regulation of anti-apoptotic proteins in stem cells is especially important in diseases such as cancer. Many cancers such as leukemia, glioblastoma, and breast and skin cancers are known to arise from cancer stem cells, which are capable of forming new tumors (Blanpain et al., 2011; Clarke and Fuller, 2006; Reya et al., 2001). Cancer stem cells have been shown to be resistant to both radiation and chemical agent-induced apoptosis (Abdullah and Chow, 2013; Pajonk et al., 2010). This resistance reduces the efficacy of cancer treatments because even when the bulk of the tumor is reduced, the cancer stem cells survive and often reform the tumor (Yu et al., 2012). One suspected reason for apoptosis resistance in cancer stem cells could be enhanced expression of anti-apoptotic proteins (Wicha et al., 2006). Indeed, a gene expression analysis of patient-derived glioblastoma stem cells (which are $CD133^{+}$) found enhanced expression of anti-apoptotic genes such as Bcl-2, XIAP and Survivin among others, compared to the $CD133^{-}$ cells which do not form tumors (Liu et al., 2006). Similar to normal stem cells, cancer stem cells are also affected by signals from niche (Plaks et al., 2015; Schepers et al., 2015, 2013). It is possible that niche signals are responsible for this increased expression of anti-apoptotic genes in cancer stem cells; therefore, niche cells may be a better therapeutic target than cancer cells (which includes the cancer stem cells) for cancer treatment.

In conclusion our results in this study not only demonstrate the significance of niche signals in stem cell survival in normal as well as stress conditions, but also underline a non-autonomous manner in which somatic support cells can lead to the death of adjacent germ cell cluster.

Materials and methods

Fly stocks and cultures

Flies were raised on standard yeast medium at 25 °C unless otherwise noted. The following stocks were used: *Bam-GFP* (kind gift of Dr. D. McKearin) (Chen and McKearin, 2003), *UASp-DIAP1* (kind gift of Dr. K. McCall) (Peterson and McCall, 2013), *Nanos-Gal4* (kind gift of Dr. E Selva) (White-Cooper, 2012), *Bam-Gal4* (kind gift of Dr. D. McKearin) (White-Cooper, 2012), *c587-Gal4* (kind gift of Dr. A. Spradling) (Skora and Spradling, 2010), *HS-Upd* (kind gift of Dr. D. Harrison) (McGregor et al., 2002), *UASt-DIAP1-RNAi* (kind gift of Dr. A. Bashirullah) (Huh et al., 2004) and *HS-DIAP1-RNAi* (kind gift of Dr. A. Bashirullah) (Yin and Thummel, 2004). Other stocks were from the Bloomington *Drosophila* Stock Center (BDSC).

Starvation

Flies were transferred to standard vials containing apple juice-agar food (Etchegaray et al., 2012) without yeast for a period of 16–24 h. Control flies were kept in identical conditions on standard yeast medium.

DIAP1 RNAi induction

Using heat shock

0–4 day old HS-DIAP1-RNAi male flies were heat-shocked in a 37 °C water bath for 1 h, returned to 25 °C for 3.5 h, and then dissected.

Using CySC and cyst cell driver

C587 Gal4;; tub-Gal80^{ts} female flies were crossed with male; UAS DIAP1 RNAi; flies and maintained at 25 °C. 0–4 day old male progeny were collected from the cross and kept at 31 °C for 6 days and then dissected and stained with TUNEL to look at dying spermatogonia.

C587 Gal4;; tub-Gal80^{ts} female flies were crossed with male; UAS DIAP1 RNAi/SM6b;UAS Dicer2 flies and maintained at 18 °C. 0–4 day old male progeny were collected from the cross and kept at 31 °C for 14 days and then dissected. Zfh-1 staining was used to count CySCs. C587 Gal4; SM6b; tub-Gal80^{ts}/UAS Dicer2 flies were used as sibling controls.

Mosaic analysis

The FLP-mediated mitotic recombination technique (Xu and Rubin, 1993) was used to generate negatively marked *thread* homozygous mutant GSC and CySC clones. Newly eclosed males of the genotype *Hs-FLP/Y; FRT2A th^5/FRT2A Ubi-GFP::nls* or *Hs-FLP/Y; FRT2A th^4/FRT2A Ubi-GFP::nls* (experimental) or *Hs-FLP/Y; FRT2A/FRT2A Ubi-GFP::nls* (control) were heat-shocked as described in Issigonis and Matunis (2012) and then dissected 2, 4, 6, 8 or 10 days after clone induction. GSC clones were identified by the absence of GFP and absence of the somatic markers Zfh1 or Traffic jam (Tj) as well as by their position adjacent to the hub. CySC clones were identified by the absence of GFP, presence of Zfh1 or Tj, and position within 2 cell diameters of the hub. Statistical analysis on the percentage of testes with clones (normalized to basal clone induction rates obtained from non-heat-shocked controls) was performed using the Fisher Exact or Chi-Squared tests.

HS-Upd induction and Stat92E^F temperature shift

To reduce JAK–STAT signaling, temperature sensitive *Stat92E^F/Stat92E^{06346}* and control *Stat92E^F/TM3* flies were raised at 18 °C; 0–4 day old adult male flies were placed at 29 °C for 24 h and then dissected. For irradiation after STAT reduction, temperature sensitive *Stat92E^F/Stat92E^{06346}* and control *Stat92E^F/TM3* flies were irradiated immediately after 24 h at 29 °C, then placed at 25 °C and dissected 3 h after irradiation. To increase JAK–STAT signaling, *HS-Upd/Y* and control *FM7/Y* adult male flies were heat shocked in a 37 °C water bath for 45 min, returned to 25 °C for 30 min, and then dissected.

γ-Irradiation

0–4 day old adult male flies were kept in a vial with standard yeast medium and exposed to a 25 Gy or 50 Gy dose of γ-radiation in a Gammacell 3000 Elan with Cesium 137 as the radiation source. The testes were dissected out, fixed and immunostained 3 h post irradiation.

Testis immunocytochemistry

Testes were dissected, fixed, and immunostained as described in Matunis et al. (1997). The following primary antibodies were used: rabbit anti-Vasa at 1:200 (Santa Cruz Biotechnology); mouse anti-DIAP1 at 1:100 (kind gift of Dr. B. Hay); rabbit anti-cleaved Dcp-1 at 1:100 (Cell Signaling); guinea pig anti-Zfh-1 at 1:500; chicken anti-GFP at 1:10,000 (Abcam); and guinea pig anti-Traffic Jam at 1:10,000 (kind gift of Dr. D. Godt). Alexa fluor-conjugated secondary IgG (H+L) antibodies (Molecular Probes/Invitrogen) were used at 1:200. Nuclei were counterstained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Roche Molecular Biochemical). Stained testes were mounted and imaged in Vectashield (Vector Labs).

Image analysis for fluorescence quantification

Confocal images were acquired in the linear range for intensity for all channels on a Zeiss LSM 5 PASCAL microscope or on a Zeiss LSM 700 microscope. ImageJ (Schneider et al., 2012) was used to identify the optical section of each testis where the hub had the largest diameter and to measure the DIAP1 fluorescence intensity in GSCs and CySCs in that section. GSCs were identified as Zfh1-negative cells next to hub, and CySCs were identified as Zfh1-positive cells within two cell diameters of the hub. Since the hub has very low amounts of DIAP1, we used the fluorescence intensity of the hub as background and subtracted it from the intensity measurement in each stem cell. We then normalized the intensity measurement for each stem cell by comparing it to the DAPI fluorescence intensity for the same cell using a similar approach as published (Nguyen et al., 2015; Starz-Gaiano et al., 2008).

Cell death analysis

Specification of the zones. This analysis was performed using flies expressing the fusion protein Bam-GFP. The testis apex was divided into a stem cell zone and a differentiating cell zone. The stem cell zone was defined as all cells within the first two tiers of nuclei surrounding the hub, and includes GSCs and CySCs and some of their immediate daughters. The differentiating cell zone was defined as all cells outside the stem cell zone and up to the posterior end of the Bam-positive zone, and includes mostly 2, 4, and 8-cell spermatogonial cysts.

Detection of apoptosis. To detect apoptotic cells, we used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Testes were fixed for 20 min in 4% formaldehyde in PBX (Matunis et al., 1997), washed 2 × 10 min in PBX, and labeled according to the manufacturer's instructions with the Millipore Apoptag kit S7165.

Quantification. To quantify apoptotic cells, we counted TUNEL-positive spots in the stem cell and differentiating cell zones. To quantify

dying spermtogonia, we counted only those TUNEL-positive spots in the differentiating cell zone that had a diameter ≥ 5 μm.

Statistical analysis

All statistical tests were performed using Prism 6 (GraphPad Software).

Author contributions

S.H., P.H. and E.M. designed the experiments. S.H. and P.H. performed experiments and data analysis. S.H. and E.M. wrote the manuscript.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.04.017.

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